RAPD TECHNIQUE USED IN ANALYZING THE GENETIC STRUCTURE OF *CYPRINUS CARPIO* SPECIES – GALITIAN AND LAUSITZ VARIETIES

R.G. Oroian, T.E. Oroian, Crina Teodora Carsai, Viorica Cosier, L. Sasca

University of Agricultural Sciences and Veterinary Medicine,
Faculty of Animal Husbandry and Biotechnologies Cluj-Napoca, Romania
e-mail: oroianrg@yahoo.com

Abstract

The purpose of this study is to analyze the genetic structure of Cyprinus carpio species, Galitian and Lausitz varieties, from Arinis fishery, Maramures county, using RAPD technique. This work perfomed to improve the artificial reproduction, by making the pairing management using DNA fingerprinting. This manual labor was applied for all the reproduction individuals from the enlarged selection nucleus (150 individuals, 60 from each variety), with the ages between three and six summers. We present in the paper the data from 6 reproduction individuals used for reproduction in 2008. We gathered the biological samples from fish fins and scales, extracted the DNA, using phenol-chloroform method, verified the DNA purity and applied RAPD technique. From a total number of 50 RAPD primers used, only 3 from Operon series (OPM-14, OPM-12 and OPQ-09) allowed the observation of electrophoretic profiles of the amplified samples. We also made the similarity matrix of the two varieties, considering the line number, calculated based on Nei Lee-Dice similarity coefficient. Based on the existent similarity, we performed the UPGMA type dendrogram, represented by two base clusters, where are included the two varieties' genotypes.

Key words: Cyprinus carpio, RAPD, DNA, dendrogram

INTRODUCTION

The analyses of carp species genetic structure are useful for the establishing of possible phenotypical correlations. The most frequent techniques used in genotyping different fish species are RAPD and microsatellite (Bartfai, R. and col.).

MATERIAL AND METHODS

DNA isolation from fish fins – K proteinase method

One cm^2 from fish fins is overnight incubated (for 10 hours), with K proteinase at 55°C. The tissue fragments are then introduced into an Eppendorf tube, of 1,5 ml, which contains 500 µl lysis buffer and 10 µl K proteinase (the equivalent of 200 µg from a stock solution of 10 mg/ml, which is prepared and freezed at – 20°C).

The lysis buffer contains: 50 mM KCl; 10 mM Tris-HCl; 2.5 mM $MgCl_2$; Tween 20.

After the incubation, 600 µl of phenol are added to each sample.

The tubes are homogenized by inversion for 15 minutes, then centrifuged 4 minutes at 8000 rpm. During centrifugation, new Eppendorf tubes of 1,5 ml are labelled. At the end of centrifugation, the tubes have a yellow inferior phase, which contains the organic solvent, a superior phase, which contains the DNA and an intermediate phase, where the proteins are stored.

There is separated the superior phase of each tube (approximately 700 µl), then are added 550 µl of phenol:chloroform. The tubes are shaked for 30-60 seconds, then are centrifuged for 3 minutes, labeling the new tubes. There is made a last extraction only with chloroform.

DNA is precipitated with ethanol 70%, cooled first at -20°C (two volumes), inverting a few times the tubes and making a centrifugation at 10000 rpm. The precipitated DNA is air dried and re-suspended in TE (10 mM Tris-HCl pH 8; 1 mM EDTA pH 8). After the complete

DNA dissolution, the samples are stored at -20°C.

The DNA integrity is verified after the migration in 1% agarose gel, stained with ethidium bromide, and a molecular weight marker. The total quantity of DNA, extracted from samples and its purity (RNA or proteins contamination), were established using an UV/VIS spectrophotometer (BioPhotometer Eppendorf).

The medium values obtained after the spectrophotometer readings (5 readings for each sample) are indicating a good purity and a high DNA amount for every extracted sample.

After DNA isolation and the purification of some samples with inadequate purity (genome wizard kit- Promega USA), the samples were amplified using a Termocycler (Uniequip, Germany).

PCR – RAPD reaction

RAPD (Random Amplified Polymorphic DNA) technique

RAPD technique is based on the allelic polymorphism determination concerning the amplification products obtaining or lacking, after the use of an arbitrary oligonucleotide primer, in a position allowing the amplification.

This variant of PCR technique don't involves the DNA cloning or sequencing and can detect more loci at the same time.

The method principle: The DNA isolated from an individual is amplified using a PCR reaction and arbitrary oligonucleotide primers, which will hybride the complementary sequences, when these exists.

The short primers sequences, random paired (8 – 12 base pairs) are used for DNA RAPD type amplification, usually resulting a presence/absence polymorphism in the gel. A situation like this, which allows the random amplification, in more points, can be realized at the hole genome. Because RAPD is a PCR based method, we will present some important singularities.

The changing of a single base in the genome is sufficient for inhibiting the primer alignment to that place and the fragment amplification, defined by the primer. RAPD analysis can detect the modification of a single base in the genomic DNA, in some conditions.

The length changes are rarely observed, often the polymorphism is observed when an amplified fragment can be present or absent, the RAPD polymorphism being used as a molecular marker.

RAPD markers are behaving like dominant markers when are observed in the descendants. This behaviour results from the fact that an amplified fragment is present in the gel (as a dominant A allele) or absent (as a recesive a allele).

In the offsprings analysis, a fragment is observed in a homozygote condition (similar to AA), being amplified either the DNA from one parent, or from the other parent, as well as in heterozygote condition.

The amplification protocol using RAPD method

For the individuals matching in pairs we used RAPD method, after a first phenotypical characterization of the individuals (dimension, body weight, body shape, etc.). RAPD reactions were made using as a matrix the DNA extracted by K proteinase method, in a final volume of 26 µl. Initially, there were tested 50 decamer primers from Operon series (Mycrosinth, Switzerland), which didn't produce any amplification, excepting 3 of them: OPM-12, OPM-14 and OPQ-09.

RAPD primers from Operon series have tge following nucleotide bases sequence:

OPM -14 (Sequence 5'-AGGGTCGTTC- 3')
OPM-12 (Sequence 5'-TTATCGCCCC -3')
OPQ-09 (Sequence 5'-TTATCGCCCC -3')

A single primer with 5'-3' sequence is needed for RAPD reaction, and we used the following reaction mixture, for each primer.

The primers were diluted initially, because they were sended lyophilized. The concentration used was of 20 picomoles and the primers were diluted with sterile water.

The reaction mixture for RAPD using OPM-14 primer

sterile water	- 14,8 µl
buffer	- 2,5 µl
dNTP	- 1 µl
primers	- Primer 1-2 µl
$MgCl_2$	-1,5 µl
Taq polymerase	- 0,2 µl
DNA	- 1 µl

Final volume: 26µl

The amplification thermic cycle

1 x 95^0 C	- 3 minutes
45 x 94^0 C	- 1 minute
36^0 C	- 1 minute
72^0C	- 2 minutes
72^0C	- 10 minutes

The amplified samples were migrated in 3% agarose gel and stained with ethidium bromide and also in 2% agarose gel stained with Seybr Save.

The samples were visualized using a data processing system, observing a high specific primer alignment.

The reaction mixture for RAPD using OPM-12 primer

sterile water	- 14,8 µl
buffer	- 2,5 µl
dNTP	- 1 µl
primers	- Primer 2-2 µl
$MgCl_2$	-1,5 µl
Taq polymerase	- 0,2 µl
DNA	- 1 µl

Final volume: 26µl

The amplification thermic cycle

1 x 95^0 C	- 3 minutes
45 x 94^0 C	- 1 minute
36^0 C	- 1 minute
72^0C	- 2 minutes
72^0C	- 10 minutes

From all the tested primers in the amplification reactions, the primers OPM-14, OPM – 12 and OPQ- 09 gave a good amplification, obtaining a different number of patterns between the scale and mirror carp.

For observing the similarity degree between the individuals from the two varieties, we calculated the matrix similarity based on Nei Lee-Dic similarity coefficient. For the existent similarity values, we made the UPGMA dendrogram, represented by two

base clusters, where are included the genotypes of the two analyzed varieties.

We identified the reproduction individuals, males and females, because we wanted to observe the traits transmission to the offsprings, after the genotype establishing, using RAPD technique and after the primers use for the population variability identification.

RESULTS AND DISCUSSIONS

The genetical characterization of the reproducers used in the present study

RAPD technique is a PCR based method, where there are used short length arbitrary primers and which doesn't amplifiy a known DNA sequence. Many times, RAPD mehod shows the polymorphism of more loci, which can characaterize a genome quickly.

It is used for the obtaining of informations concerning the inter- and intra-populational genetic diversity, the performing of fast genetic fingerprint, genetic mapping and for the marker saturation of a particular genome region. The isolated DNA from one individual or one group of individuals, is submitted to a PCR reaction, using a single arbitrary primer, which will attach to the complementary sequences of DNA matrix, when it exists. DNA-polymerase elongates the primers which found the complemetary sequence and attached to the matrix.

If casually two successive neighborhood primers (as usually at less then 3000 base pairs), are attaching to the matrix in opposite directions, each of them on one of the two strains, the defined fragment will be amplified.

If an individual has one of the two situses absent, the amplification will not take place and a polymorphism will be observed, at the level of the amplified fragments length. In RAPD analyses for the genetic diversity establishing, there are used arbitrary primers. These are usually formed by 6-10 nucleotides randomly paired. The G-C contetnt must be at least 40%, because those ones with less then 40% G-C content do not produce the amplification (because the hydrogen bonds are weaker and are breaking at 72-74°C, the polymerization temperature).

Initially, there were tested 50 decamer primers from Operon series (Mycrosinth, Switzerland), which didn't produce any amplification, excepting 3 of them: OPM-12, OPM-14 and OPQ-09.

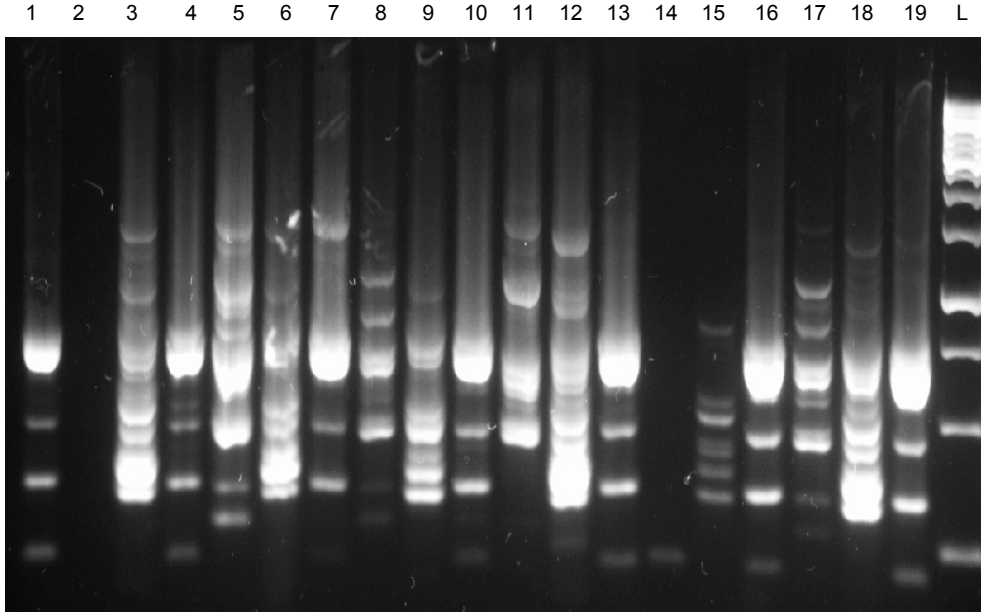

Figure 1. RAPD profile obtained with primers OPM-14,OPM-12 and OPQ-09, for the individuals from 10 breeder families of Lausitz and Galitian varieties (Ladder de 2000 pb)

The samples from 1,3,5,7,9,11positions were amplified with OPM-14 primer.

The samples from 2,4,6,8,10,12 positions were amplified with OPM-12 primer.

The samples from 13,14,15,16,17,18,19 positions were amplified with OPQ-09 primer.

The technique provides dominant markers, that is a presence/absence polymorphism, showed by the fact that an amplified fragment appears in the gel (as dominant A allele) or absent (as recessive a allele), so the dominant homozygote can't be distinguished from the heterozygote one. 95% form RAPD markers are behaving in the descendance as dominant markers and under 5% as codominant markers.

In the case of our study upon the analyzed reproducers, we obtained the amplification only with 3 primers from Operon series: OPM-14, OPM-12 şi OPQ-09.

The similarity matrix, calculated based on Nei Lee-Dic similarity coefficient is presented in table 1.

Table 1
Matrix similarity based on Nei Lee-Dic similarity coefficient at the individuals of Lausitz and Galitian varieties

No. of patterns	1	2	3	4	5	6
1	-	0,17391	0,09091	0	0,15385	0,09091
2	0,17391	-	0,45714	0,48649	0,61538	0,45714
3	0,09091	0,45714	-	0,38889	0,31579	0,58824
4	0	0,48649	0,38889	-	0,4	0,38889
5	0,15385	0,61538	0,31579	0,4	-	0,47368
6	0,09091	0,45714	0,58824	0,38889	0,47368	-

Based on the existent similarity values, we made the UPGMA dendrogram, represented by two base clusters, where are included the genotypes of the two analyzed varieties.

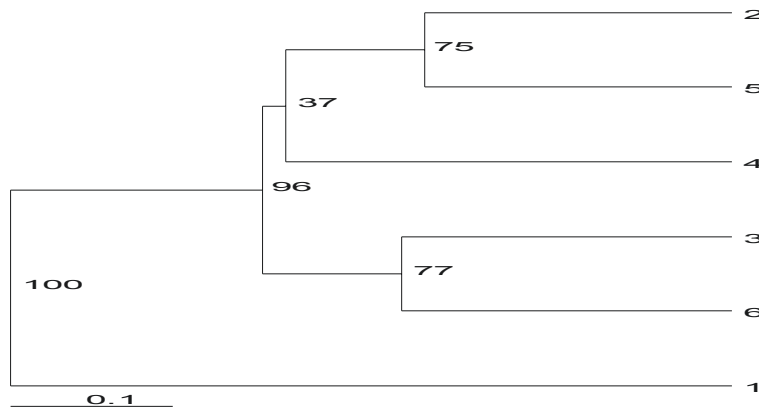

Figure 2. UPGMA type dendrogram of similarities found between the two varieties

Based on RAPD genetical analysis, we realized the genotypes distribution in the two studied varieties-*Lausitz* and *Galitian,* group shown in the obtained clusters.

The method used – UPGMA (*Unweighted Pair Group Method with Aritmetic Mean*) involves an analyzation of the group or the clustering and indicates the relatable connections between the operational

taxonomic units by similarity establishing and the difference between all the obtained polymorphisms, with primers which made the amplification.

The individuals studied formed two clusters. One cluster includes the individuals from 2 to 6 and the other cluster includes the individual 1. The cluster formed by the individuals 2-6 divides in two subgroups, one containing the individuals 3 and 6, and the other one the individuals 2, 4 and 5. Also, 2nd and 5th individuals are grouped together, separately from the 4th individual.

The results obtained after the dendrogram analysis indicate the fact that the genetic similarity of the individuals belonging to the analyzed families is variable, dependind on the geographic origin.

CONCLUSIONS

1. The DNA isolation from fish fins using K proteinase protocol is an efficient and relatively quick method, obtaining a good DNA quantity and quality.

2. From the total number of 50 decamer primers tested, belonging to Operon series (Microsynth, Switzerland), which didn't produce any amplification, excepting 3 of them: OPM – 12, OPM – 14 and OPQ – 09.

3. The UPGMA dendrogram analyses indicate the fact that the genetic similarity of the individuals belonging to the analyzed families is variable, dependind on their geographic origin.

ACKNOWLEDGEMENTS

The present study was financed from the CEEX research project, number 45/2005, Module I Biotech.

REFERENCES

Journal article

[1] Bartfai, R., Egedi, S., Yue, G.H., Kovacs, B., Urbanyi, B., Tamas, B., Gizella Tamas, Horvath, L., Orban, L., Genetic analysis of two common carp broodstocks by RAPD and microsatellite markers, Aquaculture, 2003, 219: 157-167.

Books

[2] Coşier, Viorica, Inginerie genetică, Editura Risoprint, Cluj-Napoca, 2007;

[3] Oroian, T.E., Selecţia asistată de markeri la crap, Editura Risoprint, Cluj-Napoca, 2007;

[4] Vlaic, A., Genetica peştilor, Editura Risoprint, Cluj-Napoca, 2007;